CHAPTER - 4

TO EVALUATE THE PROTECTIVE EFFECT OF
Acacia ferruginea EXTRACT AGAINST ACETIC ACID INDUCED
ULCERATIVE COLITIS

4.1 INTRODUCTION

Ulcerative colitis is an autoimmune disease in humans characterized by chronic inflammation in the alimentary tract (Podolsky, 2002). Although its etiology is unclear, it has been reported that immune dysfunction plays a decisive role in the development of UC. Moreover, there is convincing evidence that, imbalances between pro-inflammatory cytokines include tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1beta (IL-1β), IL-6, and IL-12, and anti-inflammatory cytokines such as, IL-4, IL-10, IL-11 are believed to play a crucial role in modulating inflammation (Ardizzone and Porro, 2005). Therapeutic agents for IBD include anti-inflammatory agents (5-aminosalicylates) along with some immunomodulators (6-mercaptopurine) were used. Although these treatments are effective, it associated with severe adverse effects like diarrhea, cramps, abdominal pain accompanied by fever and high blood pressure (Xu et al., 2004). Therefore, there is need to develop new therapeutic possibilities with low/nil toxicity and minimal side effects.

In traditional system of medicine A. ferruginea bark decoction in conjunction with ginger is used as an astringent for the teeth (Suresh and Rao, 1999), also as anti-diarrhoeal, for the treatment of mucous discharges, to treat itching, hemorrhages, stomatitis, irritable bowel syndrome, antileprotic drug
(Parrotta, 2001; Kirtikar and Basu, 2003) and also used to treat skin disease (Das et al., 1983). To our knowledge, very few experimental studies have been assessed in association with pharmacological aspects of A. ferruginea. Earlier studies from our laboratory showed the major phytochemicals present in the A. ferruginea extract and also potent antioxidant activity of A. ferruginea extract. Recent report showed ethyl acetate fractions of A. ferruginea stem bark exert protection against gastric ulcer in rats (Sowndhararajan and Kang, 2013). A model of acetic acid induced ulcerative colitis in rats is one of the common model used to study the pathogenesis and pathophysiology of the inflammatory bowel disease, it resembles human ulcerative colitis in histology, eicosanoid production and excessive oxygen-derived free radicals release by inflamed mucosa (Okabe and Amagase, 2005). In view of the above findings, in the present study, we made an attempt to evaluate the protective effect of A. ferruginea against acetic acid-induced ulcerative colitis in wistar rats.

4.2. Materials and Methods

4.2.1 Chemicals and reagents

Sulfasalazine was procured from Wallace pharmaceuticals Goa, India. COX-2 and iNOS polyclonal antibody were purchased from Abcam, USA and Thermo scientific, USA respectively. Lactate dehydrogenase (LDH) assay kit was procured from Biovision, USA. Myeloperoxidase (MPO), Tumor necrosis factor (TNF-α), Inducible nitric oxide synthase (iNOS) kits were obtained from USCN life science, USA. Interleukins, IL-1 beta and IL-6 were purchased from KOMA Biotech, Korea and COX-2 kit from Shangai Bluegene Biotech, China. All other chemicals used were analytical grade.
4.2.2 Collection of plant material

The fresh aerial parts of the plant were collected from Coimbatore, India. The plant was identified and authenticated at Botanical Survey of India, Coimbatore (No: BSI/SRC/5/23/2011-12/Tech-687). A voucher specimen was retained in the Department of Biotechnology, Karunya University, Coimbatore. The harvested plant samples were washed thoroughly with water and shade-dried at room temperature.

4.2.3. Preparation of extract

The shade-dried aerial parts of the plant were subjected to mechanical size reduction. The powdered material (≈ 25 g) was then extracted with methanol in a Soxhlet apparatus. Traces of solvent were then removed by evaporation and the final extract concentrated in a vacuum rotary system; the percentage yield of extract was 12% [w/w]. Based on toxicity studies, a dose of 10 mg/kg B.wt. was found to be non-toxic and selected for use in the current experiments. For each exposure, the extract was re-suspended in 1% gum acacia for subsequent administration to the mice.

4.2.4 Animals

Male Wistar rats (7-8 weeks of age, 150-180 g) were obtained from the College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (Mannuthy, Kerala, India). All rats were maintained in a controlled sterile environment maintained at a constant temperature (24±2°C), 50% relative humidity, and a 12-hr light/dark cycle. All rats had ad libitum access to standard diet pellets (Sai Durga Feeds, Bangalore, India) and filtered water. After 2 weeks of acclimatization, the rats were randomly allocated into
respective groups. All animal experiments were performed after obtaining approval from Institutional Animal Ethics Committee, Karunya University (Approval No: IAEC/KU/BT/2012/001).

4.2.5 Experimental design

Rats were divided into 4 groups, each consisting of 12 animals. The group I used as normal control, Group II, III and IV were subjected for the induction of ulcerative colitis by intra-colonic injection of 2 ml of 3% acetic acid. Group II served as ulcerative colitis control group. Group III was treated with standard drug sulfasalazine (100 mg/kg B.wt.) (Kannan and Guruvayoorappan, 2013) intra-peritoneally and Group IV was treated with A. ferruginea extract (10 mg/kg B.wt.) intra-peritoneally for 5 consecutive days before induction of ulcerative colitis with acetic acid.

4.2.6 Induction of experimental colitis in rats

All animals were kept fasting overnight, with access to water ad libitum and anesthetized by ether inhalation before induction of colitis. 2 ml of acetic acid (3%, v/v, in 9% saline) were infused for 30 s using a polyethylene tube (2 mm in diameter), inserted through rectum into the colon up to a distance of 8 cm. 24 h later, blood and colon were collected after sacrificing the animals. For separation of serum, blood samples were centrifuged at 3500 rpm (10 min) and the serum collected were stored at -20°C. Portions of colonic specimens were dissected out, washed with ice cold PBS (pH 7.2) and kept in 10% formalin for macroscopic, histopathology and immunohistochemistry studies. The remaining portions of colonic specimens were used for biochemical studies (Jagtap et al., 2004).
4.2.7 Assessment of colitis

4.2.7.1 Determination of the effect of *A. ferruginea* on macroscopic scoring during Ulcerative colitis

The severity of colitis was evaluated by an independent observer who was blinded to the treatment. For each animal, the distal 10 cm portions of the colon were removed and cut longitudinally, cleaned with physiological saline to remove fecal residues. Macroscopic inflammation scores are assigned based on the clinical features of the colon using an arbitrary scale ranging from 0-10 as follows: 0 = no damage, 1 = focal hyperemia (water oozes out), 2 = ulcerization without hyperemia or bowel wall thickness, 3 = ulcerization with inflammation at one site, 4 = ulcerization with inflammation at two sites, 5 = major sites of inflammation > 1 cm along the organ with redness, 6 = major sites of inflammation > 2 cm along the organ with redness, 7 = major sites of inflammation > 3 cm along the organ with redness, 8 = major sites of inflammation > 4 cm along the organ with redness, 9 = major sites of inflammation > 5 cm along the organ with redness and bleeding, and 10 = major sites of inflammation > 6 cm along the organ with redness, swelling, and bleeding (Jagtap et al., 2004).

4.2.7.2 Determination of the effect of *A. ferruginea* on Colon wet weight and spleen weight during Ulcerative colitis

The wet weight of the colon was assessed by the following method. A 5-cm segment of the distal colon, 3 cm proximal to the anus was resected, the lumen rinsed with ice-cold saline and weighed. Results are expressed as mg/cm of wet weight of colon, mean±SD from six samples of each group. The spleens were also obtained from animals in each group and their weight was measured.
4.2.7.3 Determination of the effect of *A. ferruginea* on colonic vascular permeability

In separate experiments, the vascular permeability of the colon was measured as described by Rivera and Stachura, (1992). Separately, six animals from each group were anesthetized, a femoral vein catheter was inserted, 1% Evans blue in 0.9% saline was injected in a volume of 0.2 ml/100g B.wt. and 30 min later, the rats were sacrificed by decapitation. The colons were removed, opened longitudinally and rinsed in saline. Approximately 200 mg segments (colon) were taken, weighed and put into formamide in a shaking bath at 37°C for 48 h. The amount of Evans blue extracted into formamide from the tissue sample was measured spectrophotometrically at 620 nm. The amount of extravasated dye was calculated from the standard curve. Tissue Evans blue concentrations were expressed as microgram per gram wet weight.

4.2.7.4 Determination of the effect of *A. ferruginea* on NO, LPO, SOD and GSH during Ulcerative colitis

Sample from the colon were homogenized in 10mmol Tris-Hcl buffer (pH 7) and the homogenate were used for the measurement of Nitric oxide (Green et al., 1982); lipid peroxidation (Ohkawa et al., 1979); glutathione (Moron et al., 1979); superoxide dismutase (Kakkar et al., 1984). The blood samples were collected separately in non-EDTA coated tubes used for separation of serum to determine serum NO level.
4.2.7.5 Determination of the effect of *A. ferruginea* on cytokines (TNF-α, IL-1β and IL-6 level), inflammatory mediators (iNOS, and COX-2 level), colonic MPO activity and serum LDH level

Colon sample from each animals were collected, weighed and tissue homogenate were prepared using Tris-Hcl buffer (pH 7), centrifuged at 500 × g for 10 min and the supernatant used for the measurement of TNF-α, iNOS, MPO (USCN Life Science, USA), IL-1β, IL-6 (Koma Biotech, Korea), COX-2 (Shanghai Bluegen Biotech, China) using standard sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit specific for murine cytokines according to the manufacture’s instruction. The blood samples collected from all animals were centrifuged, serum separated and used to determine Lactate dehydrogenase (LDH) activity using ELISA kit (Biovision, USA).

**4.2.7.6 Preparation of Nuclear Extracts**

Colon tissues were homogenized in cold PBS, and then centrifuged at 500 × g for 5 min at 4°C. The resulting supernatants were discarded. The cell pellet was resuspended in ice cold cell lysis buffer (200 µL; pH 7.9) containing HEPES (10 mM), MgCl₂ (1.5 mM), Kcl (10 mM), phenylmethyl sulfonyl fluoride (1 mM), dithiothreitol (DTT) (1 mM), Nonidet P40 (0.5%) and EGTA (1 mM) followed by centrifugation at 500 × g for 15 min. The cell pellet was resuspended in double the volume of lysis buffer and the cells were disrupted by repeated single rapid stroke using a sterile syringe. The nuclear pellet was resuspended in extraction buffer (200 µL) containing HEPES (20 mM), glycerol (25%), MgCl₂ (1.5 mM), NaCl (420 mM), PMSF (0.1 mM) and DTT (1 mM) and incubated in ice for 30 min. The nuclear suspension was centrifuged at 500 × g for 15 min at 4°C and the supernatant (nuclear extract) was frozen in aliquots at -80°C for the transcription factor profiling.
4.2.7.7 Transcription factor profiling NF-κB (p65/p50)

Transcription factor profiling was done using the Cayman™ transcription factor kit (Cayman™, Michigan, USA). The kit provided rapid, high-throughput detection of specific transcription factors, namely subunits of NF-κB such as p65 and p50. Using an ELISA based format, the transcription kit detected the DNA bound transcription factors. Bound transcription factors in the DNA were detected by specific primary antibodies towards NF-κB p65 and NF-κB p50 subunits. A horseradish peroxidase-conjugated secondary antibody was then used to detect the primary antibody. The enzymatic product was then measured using an ELISA reader and the percentage inhibition was calculated using the formula: 100 – ([OD of treated/ OD of control] x 100), where OD is optical density.

4.2.7.8 Histopathological assessment of colitis

Colonic specimens were fixed in 10 % formalin in phosphate buffered saline, embedded in paraffin, after several steps to induce dehydration in alcohol, sections of 4-μm thickness were prepared and stained with haematoxylin and eosin (H&E). Thereafter, histopathological analysis was carried out using a EVOS-xl CORE light microscope (AMG, Bothell, WA). All samples were analyzed in a blinded manner. A certified histopathologist performed all analyses/interpreted the observed outcomes.

4.2.7.9 Immunohistochemical study

The colonic tissues were fixed in 4% neutral formalin, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 µm thick) were mounted on slides, cleaned, and hydrated. The sections were treated with a buffered blocking solution (3% bovine serum
albumin in phosphate-buffered saline (PBS) for 15 min. Then, the sections were co-incubated with primary antibody for COX-2 or iNOS at a dilution of 1:400 in PBS (v/v), at room temperature for 1 and 24 h respectively, followed by washing with PBS and co-incubated with secondary antibody at a dilution of 1:500 in PBS (v/v), at room temperature for 1 h. Thereafter, sections were washed as before and with Tris-HCl 0.05 M, pH 7.66, and then co-incubated with a 3,3’-diaminobenzidine solution in darkness, at room temperature for 10 min. The sections were washed with Tris-HCl, stained with haematoxylin according to standard protocols, mounted with glycerin and observed using an EVOS-xl CORE light microscope (AMG, Bothell, WA).

4.2.7.10 Statistical analysis

All values are expressed as mean ± SD. Statistical analysis were performed using a one-way analysis of variance (ANOVA) followed by Dunnett’s test, using Graphpad InStat version 3.0, (GraphPad Software, San Diego, CA). Results from rats with ulcerative colitis treated with A. ferruginea and sulfasalazine were considered statistically significant (p values, p < 0.05) compared to those from ulcerative colitis control hosts.
4.3 RESULTS

4.3.1 Effect of *A. ferruginea* on clinical and macroscopic evaluation of the colonic lesions, colon wet weight and spleen weight

Treatment with acetic acid elicited severe macroscopic edematous inflammation in the colon 24 h after administration, as evidenced by the high score of colonic damage with increased colon wet weight. *A. ferruginea* extract ameliorated the acetic acid effect by significantly reduced the increase in colon wet weight and spleen weight. Figure 4.1 and 4.2 shows the effect of acetic acid, *A. ferruginea* and sulfasalazine treatment, in comparison with naïve controls on macroscopic examination, colon wet weight and spleen weight.

**Figure 4.1** Representative macroscopic appearance of rat colon from each group.
Figure 4.2 Effect of *A. ferruginea* on macroscopic evaluation of the colonic lesions, colon wet weight and spleen weight.

Treatments were administered once daily for 5 consecutive days before induction of colitis. **Values shown are mean± SD. Values significantly different from ulcerative colitis (no-drug-treated) control.**
4.3.2 Effect of *A. ferruginea* on colonic vascular permeability during ulcerative colitis

Vascular integrity was assessed by administration of Evan’s blue in the colon. Increase in vascular permeability was observed 30 min after exposure of acetic acid. Maximal extravasation (61.90 ± 4.12 µg/g wet weight of colon) was observed in rats with ulcerative colitis (untreated) and administration of *A. ferruginea* extract and sulfasalazine reduced significantly up to the value 33.21 ± 3.48 and 30.51 ± 2.49 µg/g wet weight of colon respectively (Figure 4.3).

![Graph showing effect of A. ferruginea on colonic vascular permeability](image)

**Figure 4.3** Effect of *A. ferruginea* on colonic vascular permeability of rats with acetic acid-induced ulcerative colitis.

Values shown are mean± SD. **Values significantly different from ulcerative colitis (no-drug-treated) control.
4.3.3. Effect of *A. ferruginea* on GSH, SOD, LPO and NO during ulcerative colitis

Acetic acid-induced colitis resulted in increased nitric oxide level in both serum and tissue (colon) in comparison with naive controls. Administration of *A. ferruginea* extract and sulfasalazine showed a significant reduction in nitric oxide level compared to acetic acid-induced colitis group. Regarding colonic non-enzymatic GSH and enzymatic SOD, colitis group exhibited a significant decrease in GSH (763.78 ± 28.49 nmol/g of wet tissue) and SOD (84.18 ± 4.92 U/mg protein) activity; however these levels were reversed in *A. ferruginea* treated animals (1105.24 ± 61.26 nmol/g of wet tissue and 104.28 ± 6.48 U/mg protein respectively) towards the value of naïve controls (Figure 4.4). Sulfasalazine also produced similar effects. In case of lipid peroxidation, acetic acid alone treated animals displayed significant increase (8.19 ± 0.64 nmol/mg protein), whereas treatment with *A. ferruginea* and sulfasalazine exhibited significant decrease in level of lipid peroxidation (4.21 ± 0.31 and 3.92 ± 0.78 nmol/mg protein respectively).

4.3.4 Effect of *A. ferruginea* on cytokines (TNF-α, IL-1β and IL-6) and inflammatory mediators (iNOS and COX-2) levels during ulcerative colitis

As illustrated in Figure 4.5, acetic acid-induced colitis group shown increased colonic TNF-α, IL-1β and IL-6 level (129.42 ± 6.13, 88.54 ± 8.84 and 116.38 ± 4.11 pg/mg tissue respectively) compared with naive controls (29.37 ± 1.48, 35.78 ± 4.93 and 31.38 ± 2.16 pg/mg tissue respectively). This increase in colonic TNF-α, IL-1β and IL-6 level were significantly attenuated by treatment with *A. ferruginea* compared to acetic acid-induced colitis group (46.29 ± 2.59, 52.49 ± 3.95 and 52.34 ± 6.23 pg/mg tissue) respectively. Sulfasalazine treated animals also produced similar results regarding cytokine levels. Colonic iNOS
and COX-2 level were significantly higher (74.97 ± 3.83 and 91.19 ± 5.99 ng/mg tissue) than value of naïve controls (12.94±0.78 and 22.47 ± 3.68 ng/mg tissue) respectively. Treatment with A. ferruginea significantly decreased these inflammatory mediators level (27.92 ± 1.48 and 49.29 ± 7.93 ng/mg tissue) respectively. Standard drug sulfasalazine also produced a marked decease compare to acetic acid-induced colitis group. The effect of A. ferruginea on inflammatory mediator’s iNOS and COX-2 level (Figure 4.6).

4.3.5 Effect of A. ferruginea on colonic MPO activity and serum LDH levels during ulcerative colitis

MPO level (indicator of colonic infiltration) in acetic acid-induced colitis group shown significant increase up to 92.48 ± 4.48 ng/g tissue. The treatment with A. ferruginea or sulfasalazine decreased significantly the MPO value (33.56 ± 2.36 and 27.54 ± 3.63 ng/g tissue respectively) towards the value of naive controls (20.16 ± 0.92 ng/g tissue). Similarly, serum LDH activity was significantly elevated after exposure of acetic acid (2103.63 ± 142.82 U/L) in comparison with naive controls (595.42 ± 61.59 U/L). A. ferruginea treatment reduced the increase in serum LDH level (773.28 ± 96.34 U/L), whereas standard drug sulfasalazine was exhibited (737.58±81.57 U/L) (Figure 4.7).
Figure 4.4 Effect of *A. ferruginea* on oxidative stress markers colonic Glutathione (GSH), Superoxide dismutase (SOD), Lipid peroxidation (LPO) and Nitric oxide (NO) in both tissue and serum during ulcerative colitis.

Values shown are mean± SD. **Values significantly different from ulcerative colitis (no-drug-treated) control.
Figure 4.5 Effect of *A. ferruginea* on colonic Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-1beta (IL-1β) and Interleukin-6 (IL-6) level of rats with acetic acid-induced ulcerative colitis.

Treatments were administered once daily for 5 consecutive days before induction of colitis. Values shown are mean± SD. **Values significantly different from ulcerative colitis (no-drug-treated) control.
Figure 4.6 Effect of *A. ferruginea* on colonic Inducible Nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX-2) level of rats with acetic acid-induced ulcerative colitis.

Treatments were administered once daily for 5 consecutive days before induction of colitis. Values shown are mean± SD. **Values significantly different from ulcerative colitis (no-drug-treated) control.
Figure 4.7 Effect of *A. ferruginea* on colonic Myeloperoxidase (MPO) and serum Lactate dehydrogenase (LDH) activity of rats with acetic acid-induced ulcerative colitis. Treatments were administered once daily for 5 consecutive days before induction of colitis. Values shown are mean± SD. **Values significantly different from ulcerative colitis (no-drug-treated) control.
4.3.6 Effect of *A. ferruginea* on Transcription factor profiling NF-κB (p65/p50) during ulcerative colitis

DNA-bound transcription factor NF-κB (p65/p50) was detected by the primary antibody. A horseradish peroxidase-conjugated secondary antibody was then used to detect the primary antibody. The enzymatic product was measured using ELISA reader. Transcription factor profiling revealed *A. ferruginea* extract could inhibit the activation and nuclear translocation of NF-κB subunits, p65 (72.37%), p50 (58.74%), similarly, reference drug sulfasalazine also exhibited p65 (73.66%) and p50 (66.57%) respectively (Table 4.1).

**Table 4.1** Effect of *A. ferruginea* on the translocation of transcription factors NF-κB (p65/p50) subunits.

<table>
<thead>
<tr>
<th>Transcription Factors</th>
<th>Ulcerative colitis control (OD)</th>
<th>Ulcerative colitis + Sulfasalazine (OD)</th>
<th>Ulcerative colitis + <em>A. ferruginea</em> (OD)</th>
<th>Percentage inhibition by Sulfasalazine treatment</th>
<th>Percentage inhibition by <em>A. ferruginea</em> treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB p65</td>
<td>0.619±0.037</td>
<td>0.163±0.014</td>
<td>0.171±0.011</td>
<td>73.66%</td>
<td>72.37%</td>
</tr>
<tr>
<td>NF-κB p50</td>
<td>0.703±0.083</td>
<td>0.235±0.036</td>
<td>0.290±0.064</td>
<td>66.57%</td>
<td>58.74%</td>
</tr>
</tbody>
</table>

NF-κB = Nuclear Factor Kappa-B, OD is optical density. Nuclear extracts from colonic tissue were prepared separately and subjected for transcription factor assay as per manufacturer’s instruction using Cayman™ (Michigan, USA) transcription factor kit. Values shown are mean±SD.
4.3.7 Histopathological assessment

Histopathological analysis of colonic sections was used to assess the protective effect of *A. ferruginea* extract on ulcerative colitis (Figure 4.8). Acetic acid administration triggered transmural necrosis in the layers of bowel wall, infiltrate consisted of mixed inflammatory cells in the mucosa, edema and loss of epithelial cells were observed in colitis group. Treatment with *A. ferruginea* significantly attenuated these signs of cell damage include, reduction in mucosal injury, edema and reduced infiltration of inflammatory cells in comparison with the colitis group.

4.3.8 iNOS and COX-2 immunostaining

Immunohistochemical examination revealed that iNOS and COX-2 expression was up-regulated in acetic acid-induced colitis group and was localized in the cells of surface epithelium and infiltrated inflammatory cells. Treatment with *A. ferruginea* or sulfasalazine reduced the overexpression of iNOS and COX-2 induced by exposure to acetic acid. In naive controls, COX-2 expression was scarcely found in the surface epithelium, mononuclear cells of lamina and propria of mucosa. Animals treated with *A. ferruginea* extract or sulfasalazine showed lowered level of iNOS and COX-2 expression in acetic acid induced colitis group (Figure 4.9 and 4.10).
**Figure 4.8** Effect of *A. ferruginea* on colon histology.

Histological colonic mucosal sections of normal rat (A), showing normal mucosa with intact epithelial surface and acetic acid-induced colitis, (B) showing massive necrotic destruction of epithelium. (C) Pre-treatment with sulfasalazine (100 mg/kg B.wt.) or (D) *A. ferruginea* (10 mg/kg B.wt.) attenuated the extent and severity of cell damage.
Figure 4.9 Immunohistochemical localization of iNOS during ulcerative colitis.

(A) Immunohistochemical localization of iNOS in normal control, (B) Positively stained brown granules for iNOS were significantly increased in both number and intensity in colonic tissue of acetic acid treated rats. (C & D) A. ferruginea (10 mg/kg B.wt.) and sulfasalazine (100 mg/kg B.wt.) treated reduced colonic iNOS expression of acetic acid treated rats.
Figure 4.10 Immunohistochemical localization of COX-2 during ulcerative colitis.

Immunohistochemical localization of COX-2 in normal control. (B) Positively stained brown granules for COX-2 were significantly increased in both number and intensity in colonic tissue of acetic acid treated rats. (C & D) *A. ferruginea* (10 mg/kg B.wt.) and sulfasalazine (100 mg/kg B.wt.) treated reduced colonic COX-2 expression of acetic acid treated rats.
4.4 DISCUSSION

Ulcerative colitis is characterized by confluent mucosal inflammation of the colon at the anal verge and extending proximally for a variable extent includes proctitis, left-sided colitis or pancolitis (Kishor et al., 2012). The pathogenesis and treatment of UC remain poorly understood. Several natural product drugs of plant origin have been proposed for use against inflammation and ulcerative colitis. To validate this hypothesis, recently a study from our laboratory revealed that the protective effect of *B. tomentosa* against experimental ulcerative colitis in rats (Kannan and Guruvayoorappan, 2013). In the current study, we provide evidence that *A. ferruginea* extract have potent anti-ulcer activity against acetic acid-induced experimental ulcerative colitis in wistar rats.

Our phytochemical screening of *A. ferruginea* extract in previous study demonstrated that, the extract is rich in polyphenols (flavonoids and phenolics), steroids, terpenoids, alkaloids, saponins, and tannins. GC/MS and LC/MS analysis of this extract shown quinones, quinoline, imidazolidine, pyrrolidine, pyrazole, thiazole, cyclopentenone, catechin and coumarin derivatives as major compounds present in the extract. These observations prompted us to continue our investigation towards its protective effect in rats where experimental colitis was induced by administration of acetic acid. It is well known, the major causative factors in initiation of human colitis include enhanced vasopermeability, up regulation of some cytokines and inflammatory mediators, prolonged neutrophil infiltration are involved in the induction of acetic acid-induced ulcerative colitis in murine model (Kojima et al., 2001; Elson et al., 1995).
Treatment with *A. ferruginea* extract attenuated ulcerative colitis as shown by reduction in macroscopic score (i.e. edematous inflammation), decreased colon wet weight in comparison with colitis control. Several studies revealed, splenic atrophy is associated with a high complication rate in colitis patients (Cho et al., 2011), similarly, treatment with *A. ferruginea* extract decreased the spleen weight in colitis control group and maintained nearby the value obtained in naïve controls. In addition, this treatment also exhibited prevention of increase in vascular permeability of colon caused due to acetic acid induction. Enhanced vascular permeability seems to be the rate limiting step in mucosal damage (Viera et al., 2000) and thus prevention of increase in vascular permeability of colon by treatment with *A. ferruginea* extract may play an active role in mucosal protection mechanism.

According to generally accepted mechanisms, oxidative stress plays an important role in pathophysiology of ulcerative colitis and there is direct evidence that increased production of free radicals include hydroxyl radical (OH−) generated from H2O2 and superoxide species may cause major deleterious effects include cell damage. Several exo-/endogenous defense mechanisms are available to limit the increased level of free radicals and the damage caused by them. This includes antioxidant enzymes such as superoxide dismutase and non-enzymatic compounds such as glutathione to protect the tissue against oxidative damage (Koek et al., 2011).

In our present study, we observed increase in SOD and GSH activity in the colitis group, possibly to compensate the colitis-induced oxidative injury. Several studies have shown depletion of GSH in tissue (colon) were observed, when antioxidants were neutralized by liberated oxygen derived free radicals. Further, the depletion of GSH leads to increase in MDA content, an end product of lipid peroxidation (LPO) which ultimately results in oxidative damage.
(Alzoghaibie et al., 2007). As expected in the present study, *A. ferruginea* extract was able to prevent the depletion of GSH and increase in MDA content (decreased lipid peroxidation) in the mucosa of acetic acid-induced control animals. Activated neutrophils produce superoxide anions (O$_2^-$) through NADPH oxidase, which reduces molecular oxygen to the O$_2^-$ radical through the enzyme myeloperoxidase (MPO). It is well known that MPO catalyzes the formation of potent cytotoxic oxidants such as hypochlorous acid from H$_2$O$_2$ and chloride ions (Hagar et al., 2007). MPO activity serves as an index of neutrophil activation, which was markedly reduced in animals treated with *A. ferruginea* extract. Suppression of MPO activity by *A. ferruginea* indicates that inhibition of neutrophil infiltration in the colonic mucosa (Van der Veen et al., 2009). Therefore, the increase in SOD and GSH activity, reduction in MPO and LPO activity, as well as the histopathological finding of the absence/decrease in cellular infiltration after treatment with *A. ferruginea* extract may indicate its anti-inflammatory and anti-oxidant effects in prevention of ulcerative colitis.

Catechins and coumarins (polyphenolic compounds) are plant metabolites with anti-oxidant properties, which have been evaluated in preclinical IBD models demonstrating promising results, although the mechanisms by which they achieve this beneficiary effect are still remains unclear (Dryden et al., 2007). For example, to provide direct evidence, coumarin derivatives have been shown to prevent the GSH depletion occurred as a consequence of the colonic inflammation (Luchini et al., 2008). Similarly another study shown catechins and alpha-tocopherol accelerates the healing of trinitrobenezene sulfonic acid-induced ulcerative colitis in rats (Sato et al., 1998). The results obtained from the present study indicate that *A. ferruginea* extract possess protective action against ulcerative colitis which may be related to the conservation of the colonic mucosal redox state due to the presence of catechins and coumarin derivatives in the extract.
Expression of iNOS and NO production in intestinal mucosa appear to be enhanced in ulcerative colitis condition. NO is generated in variety of cells from L-arginine pathway by three isoforms of NO Synthetase which include iNOS. Increased expression of iNOS leads to synthesis of NO in micromolar quantities, and when present in excess, these play pro-inflammatory role (Hagar et al., 2007). Previous study demonstrated use of iNOS inhibitors or iNOS knockout was found to be attenuate the severity of DSS-induced colitis in murine models (Krieglstein et al., 2001). It is well known, enzymes such as enzymes COX-2 and iNOS are predominantly expressed at sites of inflammation and activation of these enzymes will produce excessive inflammatory mediators which may leads to development of intestinal damage (Itzkowitz, 2006).

In our present study the quantitative measurement of NO and iNOS level, immunohistochemical analysis of iNOS and COX-2 indicates administration of A. ferruginea extract was found to decrease NO production through down-regulation of iNOS and also significantly diminished COX-2 expression indicate its protective role against acetic acid-induced ulcerative colitis. The cytosolic enzyme, LDH is released in to the blood stream up on cell damage during necrosis. Therefore, serum LDH activity can be used as an indicator of cell membrane integrity as well as measurement of cytotoxicity (i.e. marker of intestinal damage) (Van der Veen et al., 2009). Increase in serum LDH activity was observed in colitis group, whereas treatment with A. ferruginea extract leads to decrease in LDH activity which indicates its repair mechanism of colonic tissue damage.

TNF-α, IL-1β and IL-6 has been associated with pathogenesis of IBD by inducing the expression of different pro-inflammatory mediators (Sternlicht and Werb, 2001). TNF-α, a pleiotropic cytokine released by macrophages, involved in the inflammatory process of IBD patients and also induces the
production of other cytokines include IL-1β and IL-6 which acts as key mediators of the inflammatory cascade in IBD models. Earlier studies have shown IL-1 receptor antagonist was shown to suppress the infiltration of inflammatory cells, MPO activity of cells in areas of edema in animals with experimental colitis (Dionne et al., 1998). Similarly, IL-6 has shown to stimulate neutrophil chemotaxis and is associated with necrosis in the colon which leads to tissue destruction (Liu and Wang, 2011). In addition, our results are in agreement with report of earlier studies that, pyrrolidine dithiocarbate, a pyrrolidine derivative was able to inhibit colonic TNF-α and MPO levels in acetic acid-induced colitis in rats (Hagar et al., 2007). Interestingly, thymoquinone, a quinone derivative has been shown to protect the gastric damage in rats by inhibiting MPO levels (Arslan et al., 2005). The level of MPO, TNF-α, IL-1β and IL-6 in colonic epithelium was found to be lower in colitis-induced rats treated with A. ferruginea extract than those of sulfasalazine treated group.

Nuclear factor-κB signaling pathway plays a vital role in regulating number of genes involved in inflammatory response include pro-inflammatory cytokine production, cell survival, cell death and also in expression of COX-2 and iNOS enzymes (Malek et al., 2007). It is well known that NF-κB activation plays a key role in regulation of gene transcription of these pro-inflammatory mediators include TNF-α, IL-1β, IL-6, COX-2 and iNOS during the inflammatory process in ulcerative colitis (Zhang et al., 2009). NF-κB exists mainly as a heterodimer composed of subunits of the Rel family, p50 and p65. NF-κB localizes to the cytoplasm, where it is bound by IκB proteins in resting stage, but during inflammatory condition, IκB is phosphorylated by IκB kinase, subsequently degraded by proteasome, then get released and translocates in to nucleus, where it triggers the transcription of multiple genes involved in inflammatory cascade (Wang et al., 2011). Thus, inhibiting the NF-κB activity might help to alleviate the severity of ulcerative colitis. The present study
confirms administration of *A. ferruginea* extract could inhibit the activation and nuclear translocation of NF-κB subunits p50 and p65. These findings are supported by recent *in vitro* and *in vivo* studies revealed epigallocatechin-3-gallate (EGCG), a polyphenolic compound blocks the activation of NF-κB transcription factor in intestinal epithelial cells thereby, reduces the severity of colitis by inhibition of pro-inflammatory mediators and expression of COX-2 enzymes (Ran et al., 2008). Similarly, 4-methylesculetin, a coumarin derivative was shown to inhibit colonic oxidative stress and suppression of TNBS induced colitis (Witaicenis et al., 2012). Collectively, these results displayed that administration of *A. ferruginea* effectively overwhelms inflammatory response in the colon via inhibition of NF-κB signal transduction pathways.

In conclusion, the present investigation clearly indicates that treatment with *A. ferruginea* extract was effective in protection against acetic acid-induced ulcerative colitis in rats via modulation of oxidant/anti-oxidant balance in colonic tissue, inhibition of production of inflammatory mediators which include iNOS, COX-2, pro-inflammatory cytokines and inhibition of NF-κB signal transduction pathways. This is most likely due to high content and synergistic activity of specific constituents such as flavonoids and phenolics predominantly catechins, coumarins, quinones and pyrrolidine derivatives. Nevertheless, the precise molecular mechanism by which *A. ferruginea* extract mediates this protective action remains to be determined. Further investigations are in progress in our laboratory to isolate the specific bioactive agents in the extract with potential for use in anti-IBD therapy, and to elucidate their associated mechanism of therapeutic action.